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Metabolic engineering to produce phytochromes with phytochromobilin, phycocyanobilin, or phycoerythrobilin chromophore in *Escherichia coli*

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Abstract **By co-expression of heme oxygenase and various bilin reductase(s) in a single operon in conjunction with apophytochrome using two compatible plasmids, we developed a system to produce phytochromes with various chromophores in *Escherichia coli*. Through the selection of different bilin reductases, apophytochromes were assembled with phytochromobilin, phycocyanobilin, and phycoerythrobilin. The blue-shifted difference spectra of truncated phytochromes were observed with a phycocyanobilin chromophore compared to a phytochromobilin chromophore. When the phycoerythrobilin biosynthetic enzymes were co-expressed, *E. coli* cells accumulated orange-fluorescent phytochrome. The metabolic engineering of bacteria for the production of various bilins for assembly into phytochromes will facilitate the molecular analysis of photoreceptors.**

Keywords: Bilin reductase; Chromophore; Phycocyanobilin; Phycoerythrobilin; Phytochrome; Phytochromobilin

1. Introduction

Photosynthetic organisms utilize light both as an energy source for photosynthesis and as source of signals for photomorphogenesis, and have developed highly sophisticated systems for photoperception, energy transfer, and signal transduction. The presence of chromophores in photoreceptors is essential for photobiological reactions. Tetrapyrrole molecules including chlorophylls and phytobilins are the prosthetic groups for light perception proteins in plants and algae. Phycocyanobilin (PCB) and phycoerythrobilin (PEB) are linked to light harvesting phycobiliproteins in algae. PCB also functions as a chromophore precursor for phytochrome-like proteins in cyanobacteria, although some cyanobacterial and bacterial phytochromes use biliverdin (BV) as the chromophore. Plants possess phytochromobilin (PΦB) as a chromophore precursor for phytochromes, which are a major class of photoreceptors for regulating numerous responses to changes in wavelength, fluence, duration, and direction of light in plants.

The enzymes and genes for phytobilin biosynthesis have been identified from several photosynthetic organisms [1,2]. Heme is cleaved to BV by a ferredoxin-dependent heme oxygenase, and then further reduced by a family of bilin reductases. The gene for heme oxygenase from higher plants was first identified by positional cloning of the *Arabidopsis HY1* gene [3,4] and subsequently an enzymatic assay was performed with recombinant HY1 protein [4,5]. A cyanobacterial gene for heme oxygenase (*ho1*) from *Synechocystis* PCC6803 was also identified by genetic complementation of the *Arabidopsis hy1* mutant [6]. These enzymes are soluble and ferredoxin-dependent with weak structural similarity to microsomal heme oxygenase in animals. A gene for bilin reductase was also identified by map-based cloning of *HY2* in *Arabidopsis*. The *Arabidopsis HY2* gene was novel, and enzymatic analysis performed with recombinant protein demonstrated that it encoded PΦB synthase (PΦB:ferredoxin oxidoreductase) [7]. PΦB synthase is a ferredoxin-dependent enzyme that catalyzes the reduction from BV to PΦB. By comparative genomics and biochemical assays, genes for other bilin reductases with different substrate and double-bond specificities involved in phycobilin biosynthesis have been identified in cyanobacteria [8]. PCB is synthesized from BV by 4-electron reduction by PcyA (PCB:ferredoxin oxidoreductase). PEB is also synthesized from BV via two successive 2-electron reductions by PebA (dihydrobiliverdin:ferredoxin oxidoreductase) and by PebB (PEB:ferredoxin oxidoreductase) [8].

The gene identification of heme oxygenases and bilin reductases has now enabled to genetic engineering of bilin biosynthesis and photoreceptor reconstitution in model microorganisms. Phytochromes, which have autocatalytic lyase activity, can assemble with open linear tetrapyrroles with a ethylidene group at C3 in the A-ring as chromophores. As *Escherichia coli* cells naturally synthesize heme, it is possible to produce bilins in *E. coli* by adding two subsequent steps by genetic transformation; ring cleavage of heme by a heme oxygenase to produce BV and further reduction by bilin reductases. Co-expression of Ho1 and PcyA with cyanobacterial apophytochrome 1 (Cph1) resulted in production of holo-Cph1 in *E. coli* [9,10]. The entire pathway for a subunit for

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Abbreviations: BV, biliverdin; CBD, chitin binding domain; Cph1, cyanobacterial phytochrome 1; PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; Pfr, far-red-absorbing form of phytochrome; phyB, phytochrome B; Pr, red-absorbing form of phytochrome; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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phycocyanin was also reconstituted with PCB in *E. coli* by the similar method although the genes for the heterodimeric lyase for chromophore attachment were also included to the expression system [11].

Here, we have developed a system to produce PΦB, PCB, and PEB by using the genes for bilin reductases, *HY2, pcyA, and pebA; pebB*, respectively. Co-expression of a truncated plant phytochrome with natural chromophore PΦB and structurally related PCB will facilitate further molecular studies of plant phytochromes. Our system to express phytochrome with PEB chromophore in *E. coli* will be potentially applicable for the development of fluorescent probes known as phytofluors [12].

2. Materials and methods

2.1. Plasmid construction

The expression vectors for bilin biosynthesis were first constructed in pQE30 (Qiagen GmbH, Hilden, Germany) to provide an inducible T5 promoter, then the expression cassettes with the promoters and bilin biosynthetic genes were subcloned into pACYC184 [13], which is compatible with ColE1 plasmids. DNA fragments containing ORFs for bilin metabolic enzymes and appropriate flanking-sequences containing ribosome binding sites and restriction sites (underlined) were obtained by polymerase chain reaction (PCR) with KOD DNA polymerase (Toyobo, Osaka, Japan). The structures of the constructs for bilin biosynthesis and phytochrome expression were illustrated in Fig. 1. Outline of the constructs is as follows. The *ho1* gene (*sll1184*) for heme oxygenase [6] was PCR-amplified from genomic DNA of *Synechocystis* sp. PCC6803 with the *ho1* primer set, 5′-GGAGGAATTCTTAAGAAGGAGATATACATATGAGTGTCAACTTAGCT-3′ and 5′-GCGCTCGAGGATAAGTTGTCACGCTAGGTA-3′, and subcloned into pQE30 to give pKT210. The *pcyA* gene (*slr0116*) for PCB:ferredoxin oxidoreductase [8] was PCR-amplified from *Synechocystis* genomic DNA with the *pcyA* primer set, 5′-GCGCTCGAGTATTCCCATTGCTTTGCCCTA-3′ and 5′-CGGCTCGAGGCTAAACAACCTACGATTAGT-3′, and subcloned into pKT210 to give pKT211, in which *ho1* and *pcyA* were in tandem array. The *HY2* cDNA for PΦB synthase lacking a transit peptide (*mHY2*) was PCR-amplified from *HY2* cDNA [7] with the *HY2* primer set, 5′-GGAGCTCGAGAAGAAGGAGATATACATATGAGAGTCTCTGCTGTGTCGTAT-3′ and 5′-GGAGGTCGACCTGAGAGACTCCTCATGCTG-3′, and subcloned into pQE30 to give pKT217. The insert for the *ho1* gene from pKT210 was subcloned into pKT217 to give pKT218, in which *ho1* and *mHY2* were in tandem array. The synthetic operons of biosynthetic genes for BV, PCB, and PΦB under the control of T5 promoters in plasmids pKT210, pKT211, and pKT218, were PCR-amplified with the T5-*ho1* primer set, 5′-GTCTGCTAGCAAATCATAAAAAATTTATTTGC-3′ (T5ProNhe) and 5′-GCGGTCGACGATAAGTTGTCACGCTAGGTA-3′, the T5-*ho1-pcyA* primer set, T5ProNhe and 5′-CGGGTCGACGCTAAACAACCTACGATTAGT-3′, the T5-*ho1-HY2* primer set, T5ProNhe and 5′-GGAGGTCGACCTGAGAGACTCCTCATGCTG-3′, and replaced the tetracyclin-resistance gene at the *Xba*I and *Sal*I sites in pACYC184 to give pKT270, pKT271, and pKT272, respectively (Fig. 1). The genes *pebA* and *pebB* for dihydrobiliverdin:ferredoxin oxidoreductase and PEB:ferredoxin oxidoreductase, respectively, in *Synechococcus* sp. W8020 were PCR-amplified with the *pebA* primer set, 5′-GGAGCTCGAGGAGAAATTAACTATGATCTTTGATTCATTTCTC-3′ and 5′-GGAGGTCGACTCATTTGTGAGAGGAGGAGG-3′ and the *pebB* primer set, 5′-GGAGCTCGAGGAGAAATTAACTATGATCACAAATCAAAGATTC-3′ and 5′-GGAGGTCGACTTATAGATCAAAAAGCACAG-3′ from expression plasmids originally used in enzymatic assays [8], and cloned into pKT270 to give pKT278 (Fig. 1). The DNA fragment for *cph1* [14] was PCR-amplified from *Synechocystis* genomic DNA with the *cph1* primer set, 5′-CGCGGATCCATGGCCACCACCGTACA-3′ and 5′-TCCCCGGGTTAGTTGCCAATGGGGAT-3′, and inserted into of the *Bam*HI and *Sma*I sites of pQE30 to give pKT214. The DNA fragments for the amino-terminal region of *Arabidopsis* PHYA (N617) and PHYB (N651), were PCR-amplified by from *PHYA* and *PHYB* cDNAs [15] with the *PHYA* primer set, 5′-GGAATTCCATATGTCAGGCTCTAGGCCG-3′ and 5′-CCGCTCGAGAGCTTCTAGTTCTTGTATACC-3′, and the *PHYB* primer set, 5′-GGAATTCCATATGGTTTCCGGATGCGG-3′ and 5′-CCGGAATTCTGCACCTAACTCATCAATCCC-3′, and inserted into the *Nde*I–*Xho*I sites and *Nde*I–*Eco*RI sites of pTXB1 (New England Biolabs, Ipswich, MA, USA) to give pKT306 and pKT307, respectively (Fig. 1).

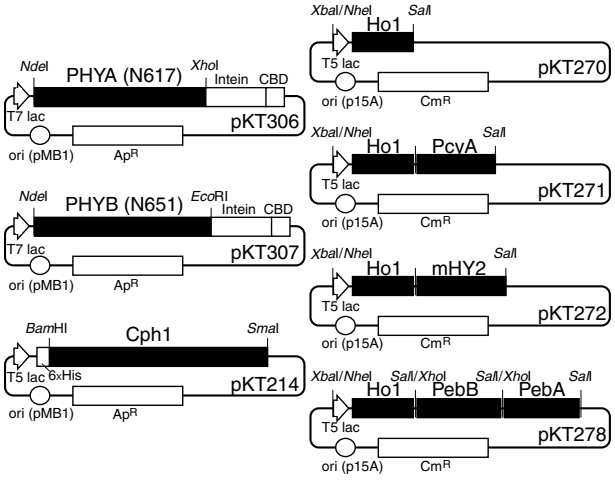


Fig. 1. Plasmid constructs for the expression of phytochrome apoproteins and biosynthesis of various chromophores in *E. coli*. Plasmids, pKT214, pKT306, and pKT307 are high-copy-number vectors for apophytochrome expression of His-tagged Cph1, chitin-binding-domain (CBD)-tagged PHYA (N617), and CBD-tagged PHYB (N651), respectively. Plasmids, pKT270, pKT271, pKT272, and pKT278 are low-copy-number vectors for the biosynthesis of BV, PCB, PΦB, and PEB, respectively. T5 lac, chimeric promoter for T5 RNA polymerase with the lac operator; T7 lac, T7 promoter with lac operator; Ap^R and Cm^R, resistance genes for ampicillin and chloramphenicol, respectively; ori (pMB1) and ori (p15A), replication origins from pMB1 and p15A, respectively.

2.2. Expression and purification of phyA (N617) and phyB (N651) with PΦB or PCB chromophore

E. coli cells ER2566 were transformed with a combination of one plasmid for bilin biosynthesis (pKT271 or pKT272) that provides resistance to chloramphenicol and a second plasmid for PHYA (N617) (pKT 306) or PHYB (N651) (pKT307) expression that provides resistance to ampicillin. Transformants selected with both antibiotics were used for the small scale primary cultures (~10 ml). Aliquots of the primary cultures were stored in the presence of 7% dimethylsulfoxide at −70 °C. The *E. coli* cells harboring both expression plasmids were grown at 37 °C with shaking at 250 rpm to an $OD_{600\,nm}$ of 0.5 in 1500 ml of LB media containing 2% glucose, 50 mg/L ampicillin, and 30 mg/L chloramphenicol. The culture was cooled to 18 °C. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. The cells were further cultured overnight at 18 °C in the dark. The cells were collected by centrifugation and resuspended in 50 ml of lysis buffer containing 50 mM Na-PO_4 pH 7.0, 100 mM NaCl, 0.1% Triton X-100, 1 mM 2-mercaptoethanol, and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and disrupted by sonication (Model UR-20P; Tomy Seiko Co., Tokyo, Japan) at 4 °C. Crude soluble extract was prepared by centrifugation at $12000 \times g$ for 10 min. The expressed phytochrome protein fused to chitin binding domain (CBD) was purified by chitin affinity chromatography using 3 ml bed volume of Chitin Beads (New England Biolabs) according to the manufacturer's instruction. The bound protein was washed and self-cleaved by incubating with the cleavage buffer containing 20 mM Tris–HCl pH 7.8, 500 mM NaCl, 0.1 mM EDTA, 50 mM DTT for 2 days at 4 °C. Further purification was performed by gel filtration chromatography (HiLoad 16/60 Superdex 200 pg, ÄKTA, Amersham Bioscience, Piscataway, NJ, USA). The incubation experiment with PCB in vitro was performed

as described previously [16] before purifying by gel filtration chromatography. PCB was prepared from *Spirulina* as described previously [17].

2.3. Expression of Cph1 with PEB chromophore in E. coli

E. coli cells JM109 harboring both pKT214 and pKT278 were selected by resistance to ampicillin and chloramphenicol. The *E. coli* cells were precultured at 37 °C with shaking at 230 rpm in the dark to an $OD_{600\,nm}$ of 0.6 in 300 ml LB medium, and then IPTG was added to a final concentration of 1 mM. The culture was further incubated overnight at 25 °C with shaking at 100 rpm in the dark. Cells were collected by centrifugation and resuspended in 10 ml of lysis buffer containing 20 mM Tris–HCl pH 7.0, 200 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Roche), and disrupted by sonication on ice. Crude soluble extract was prepared by centrifugation at $12000 \times g$ for 10 min. The resulting supernatant was loaded onto a nickel column containing 1 ml bed volume of His·Bind® Resin (Novagen, Madison, USA), and Cph1 protein was purified at 4 °C according to the manufacturer's instruction. Fluorescence images of the purified protein was recorded by digital camera (Fine Pix 4900Z, Fuji film, Tokyo, Japan), using appropriate filters for fluorescence microscopy (excitation 546 nm, beam splitter 560 nm, and emission 575–640 nm; Filter set No. 20; Carl Zeiss, Göttingen, Germany). Spectra of fluorescence and absorption of purified Cph1 protein with PEB adduct was obtained using a fluorescence spectrophotometer (F-3010, Hitachi High-Technologies, Tokyo, Japan). Monochrometers were adjusted to 2.5 nm bandpass for all measurements. Fluorescence emission spectra were obtained by excitation at 546 nm.

2.4. Fluorescence microscopy

The fluorescence emission spectrum of the *E. coli* cells was observed by fluorescence microscope (Axiophoto, Carl Zeiss) using appropriate filters (Carl Zeiss, No. 20) and images were captured with a cooled CCD camera head system (ZVS-3C75DE, Carl Zeiss).

2.5. Phytochrome detection by zinc blot and difference spectrum

For zinc blot analysis, proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) were incubated in buffer containing 100 mM zinc acetate and 150 mM Tris–HCl pH 7.0 for 10 min. Fluorescence was detected using FM-BIO II (Hitachi High-Technologies). Absorbance and difference spectra of phytochrome were obtained with a spectrophotometer (HP8453 UV–Visible system, Hewlett Packard GmbH, Waldbronn, Germany) essentially as described previously [18].

3. Results

3.1. Biosynthesis of bilins in E. coli

To express phytochrome proteins with chromophores in *E. coli*, we firstly introduced genes to biosynthesize bilins from heme into the cells. We prepared the inducible synthetic operon composed of a heme oxygenase and a bilin reductase(s) in a plasmid pACYC184, which contains the p15A origin that is compatible with ColE1 vectors and is commonly used for recombinant protein expression in *E. coli*. For the heme oxygenase, we used *ho1* (Cyanobase ID. *sll1184*) from *Synechocystis* since it is a prokaryotic gene with no coding region for a transit peptide and the heme oxygenase activity from Ho1 has been demonstrated in *E. coli* previously [9,10]. *E. coli* cells harboring the *ho1* plasmid (pKT270) turned green when heme oxygenase expression was induced, indicating accumulation of BV (data not shown). To produce various phytobilin in *E. coli*, the genes for bilins reductases with different specificities were combined with *ho1* (Fig. 1). To produce PΦB, which is a natural precursor of the chromophore for plant phytochromes, the *Arabidopsis HY2* gene that encodes PΦB synthase [7] was used after removal of the region for the transit peptide (mHY2). The *E. coli* cells harboring the plasmid containing the *ho1-mHY2* operon (pKT272) showed a pale green color when expression was induced. We detected accumulation of PΦB in cells by reverse phase HPLC analysis (data not shown). The *pcyA* gene (Cyanobase ID. *slr0116*) from *Synechocystis* sp. PCC6803 was used to produce PCB by the expression of PCB:ferredoxin oxidoreductase [8]. However, the *E. coli* cells harboring the plasmid containing *ho1* and *pcyA* (pKT271) did not show a strong color change when expression was induced. Instead, we observed that significant levels of PCB were detectable in the culture medium (data not shown).

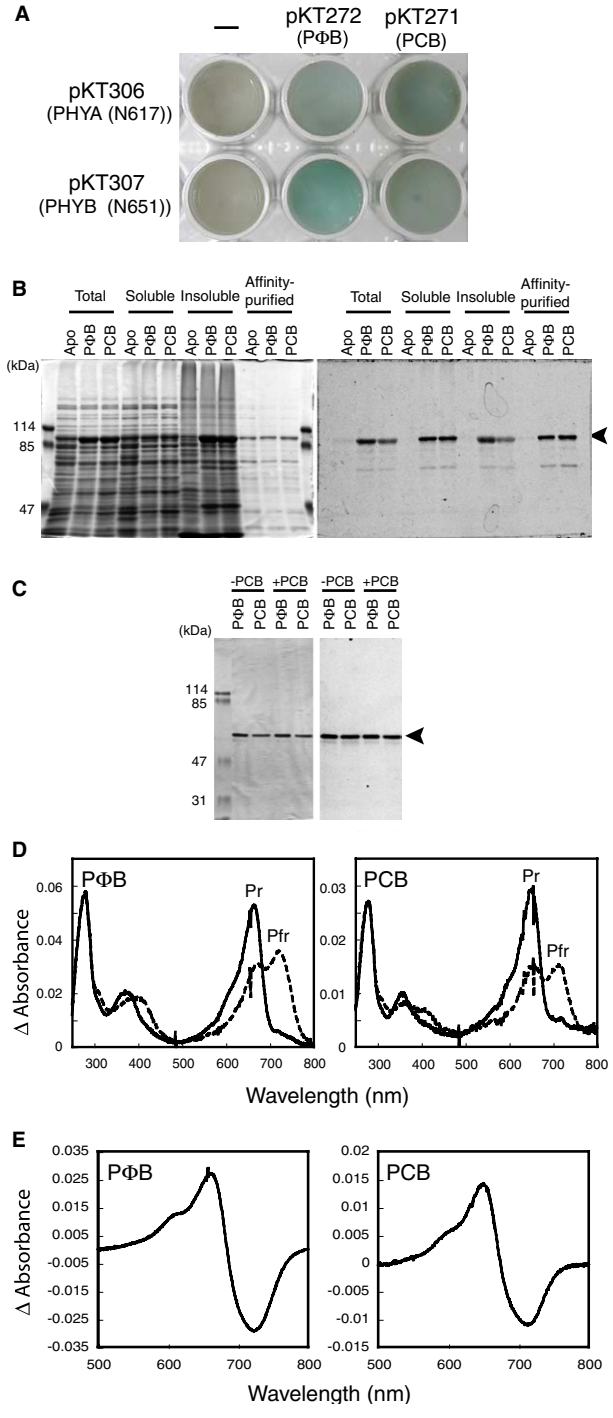
3.2. PΦB biosynthesis and recombinant phytochrome with PΦB adduct in bacteria

Expression systems for recombinant phytochrome in *E. coli* have been reported with the combination of a cyanobacterial phytochrome gene *cph1* with PCB biosynthesis genes *ho1* and *pcyA* [9,10]. As PΦB is a natural precursor of the chromophore for plant phytochromes, the truncated *Arabidopsis PHYA* (N617) and *PHYB* (N651) were co-expressed in *E. coli* with two genes, *ho1* and *mHY2,* from the synthetic PΦB biosynthesis operon in pKT272. We also prepared cells co-expressing truncated phytochromes with the operon for PCB biosynthesis for comparison. We used a photosensory domain of PHYB, PHYB (N651), as a phytochrome apoprotein since it acts as functional phytochrome B (phyB) in the nucleus in plants [19]. PHYA (N617) was also used to express a domain comparable to PHYB (N651). We induced gene expression in *E. coli* and checked for color change of the cells. The PHYA (N617) and PHYB (N651)-expressing cells co-transformed with the PΦB biosynthesis enzymes turned blue-green after induction of gene expression while those with the PCB biosynthesis enzymes turned blue (Fig. 2A).

Attachment of the bilin prosthetic group covalently to the proteins can be specifically detected by zinc blot analysis [20]. Crude protein extracts, supernatant, insoluble fraction, and affinity purified proteins from *E. coli* cultures of PHYB (N651) were analyzed by (SDS–PAGE)/Coomassie staining and zinc blot assays (Fig. 2B). Fluorescent bands in the zinc blot that corresponded to PHYB (N651) were observed only in the extracts prepared from the *E. coli* co-expressing bilins and phytochrome proteins (Fig. 2B, right). No fluorescent signals were observed in apo-PHYB (N651) expressing cells. These results indicated that PHYB (N651) expressed with bilin biosynthesis genes were covalently assembled with bilin.

As phyB (N651) proteins with bilin adducts were expressed as fusion proteins with an intein and a CBD, phyB (N651) was purified by affinity column chromatography with chitin beads and cleaved by the intein activity. Further purification was performed by gel filtration chromatography to apparent homogeneity (Fig. 2C). The absorption spectra of holo phyB (N651) after saturating illumination of red light and far-red light are shown (Fig. 2D). The difference spectrum between far-red-irradiated Pr form and red-irradiated Pfr form was detected with maxima and minima at 662 and 724 nm, respectively, in the extract from cells harboring the plasmid pKT272 for PΦB biosynthesis, and at 650 and 715 nm, respectively, in the extract from cells with the plasmid pKT271 for PCB biosynthesis (Fig. 2D and E, Table 1). The latter values were in good agreement with those for the reconstituted phyB (N651) with PCB in vitro [21]. We also analyzed spectrophotometric properties of phyA (N617), and observed photoreversibility of red-absorbing form of phytochrome (Pr) and

far-red-absorbing form of phytochrome (Pfr) forms for phyA (N617) (Table 1). Although the expression levels of phyA (N617) were lower than those of phyB (N651), the extent of chromophorylation of the recombinant proteins was as efficient as that of phyB (N651) as determined from zinc blot fluorescence levels (data not shown). The blue-shifted spectra of the PCB adduct compared with that of the PΦB adduct were reasonable from structural basis of the number of conjugate double bonds, and consistent with previous studies by in vitro reconstitution [22] and from plant extracts [18]. These results indicated that photoactive phytochromes with PΦB or PCB chromophore were synthesized in *E. coli*.



The specific absorption ratio (SAR), defined as the ratio of absorbance maxima around 655 nm and absorbance at 280 nm for the Pr form, is used to evaluate purification and chromophore binding efficiency of phytochromes [e.g. [23]]. The SAR values for Pr form of phyB (N651) proteins of PΦB and PCB adducts purified from *E. coli* were 0.96 and 1.05, respectively. Further incubation with PCB did not increase the zinc blot signals (Fig. 2C) or the SAR values (Table 1), indicating that the chromophore binding was saturated in *E. coli*.

3.3. Co-expression of phytochrome with PEB

PEB, which is a natural precursor of the phycoerythrin chromophore, has a structure similar to PΦB but lacks the C15 double bond. As a consequence, apophytochrome can bind PEB as a chromophore and gives intense orange fluorescence called phytofluor when excited [12]. To reconstitute phytofluor in vivo, we simultaneously introduced two plasmids, pKT214 to express Cph1 and pKT278 to produce PEB, into *E. coli*. The *E. coli* cells harboring both plasmids showed orange fluorescence under a fluorescence microscope after induction by IPTG (Fig. 3A). The fluorescence from *E. coli* cells was significantly stronger in the cells cultured with slow shaking (~100 rpm) than with vigorous shaking (~250 rpm) (data not shown), although we have not determined the underlying mechanism. Ubiquitous fluorescence with intense spots probably from inclusion bodies, was observed only in the co-transformed *E. coli* cells. The *E. coli* pellet was pink after centrifugation, indicating the accumulation of Cph1 protein with PEB chromophore (data not shown). In contrast, *E. coli* cells transformed with either pKT214 or pKT278 did not show any visible color change or fluorescence (data not shown). Covalent association of PEB with Cph1 was examined by zinc blot analysis. Total crude extracts from the induced *E. coli* cells were separated by SDS–PAGE. An 84-kDa band corresponding to Cph1 in the extract from the cells harboring both pKT214 and pKT278 showed a fluorescence signal in zinc blot analysis (Fig. 3B).

The Cph1 protein was affinity-purified by nickel column chromatography, as the protein was His-tagged at its amino-terminus. The purified Cph1 protein from *E. coli* was also pink

Fig. 2. Expression of truncated *Arabidopsis* phytochromes with PCB or PΦB adduct in *E. coli*. (A) *E. coli* cell cultures induced by IPTG. Photograph of microwell plates with cells harboring plasmids shown in the respective columns and rows. (B) Expression analysis of phyB (N651). SDS–PAGE/Coomassie staining (left) and zinc blot (right). Soluble and insoluble fractions were the supernatant and pellet, respectively, of the crude extracts after centrifugation. Total protein, fractionated proteins, and chitin-beads affinity-purified protein were analyzed by SDS–PAGE (7.5%). Strains harboring pKT307, pKT272 + pKT307, and pKT271 + pKT307 are indicated as Apo, PΦB, and PCB, respectively. Molecular masses of the protein standard are shown (left). An arrow indicates the position of PHYB (N651). (C) Purification of phyB (N651) with PΦB or PCB adduct by gel-filtration chromatography. SDS–PAGE/Coomassie staining (left) and zinc blot (right). Affinity-purified protein shown in (B) was digested in the presence of dithiothreitol, and eluted from chitin-beads. The eluted samples were incubated in the presence (+PCB) or absence (−PCB) of PCB, then further purified by gel filtration chromatography. (D) Absorption spectra of purified phyB (N651) with PΦB or PCB adduct after saturating irradiation with red light (Pfr) of far-red light (Pr). (E) Difference spectra of phyB (N651) purified from *E. coli*. Difference spectra of absorption spectra in (D) are shown.

Table 1
Comparison of spectroscopic and quantitative data for phyA (N617) and phyB (N651) purified from *E. coli*

Sample	Adduct	$\lambda\Delta A_{max}$ (nm)	$\lambda\Delta A_{min}$ (nm)	Yield[a] (mg)	SAR[b]	
					−PCB	+PCB
phyA (N617)	PΦB	662	728	1.8	ND	ND
phyA (N617)	PCB	652	717	1.8	ND	ND
phyB (N651)	PΦB	662	724	3.3	0.96	0.98
phyB (N651)	PCB	650	715	2.6	1.05	1.03

ND, not determined.
[a]Yield was calculated for the expected amount of phytochrome protein in 1 L cultures of *E. coli* from the protein concentrations determined by Bradford assay [29].
[b]The specific absorbance ratio (SAR) is defined in the text, and the values in +PCB samples were those obtained after further incubation of −PCB samples with PCB in vitro.

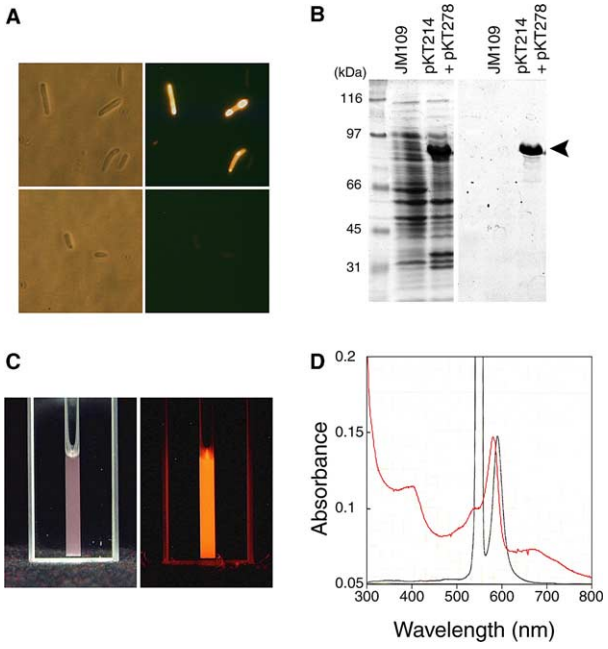


Fig. 3. Expression of Cph1 with PEB adduct in *E. coli*. (A) Micrographs of *E. coli* cells harboring both PEB biosynthesis plasmid (pKT278) and Cph1 expression plasmid (pKT214) are shown in the upper panels. As a control, those harboring only the PEB biosynthesis plasmid (pKT278) are also shown in the lower panels. Cells were observed after overnight induction by IPTG. Left, differential interference microscopy; right, fluorescence microscopy. (B) SDS–PAGE/Coomassie (left) and zinc blot analysis (right) of Cph1 with PEB adduct. Crude extracts from the host *E. coli* cells (JM109) and the cells harboring pKT214 and pKT278 after induction by IPTG were separated by SDS–PAGE (10%). (C) Affinity-purified His-tagged Cph1 protein from the cells harboring pKT214 and pKT278. The pictures were taken by flash photography (left) and fluorescence photography irradiated by excitation light. (D) Spectrophotometric analysis of Cph1 purified from cells harboring pKT214 and pKT278. Fluorescence spectrum and absorption spectrum of Cph1 protein were plotted in black and red, respectively. The scattering at 546 nm was derived from excitation light.

in solution and showed orange fluorescence when irradiated with light of 546 nm (Fig. 3C). The spectrophotometric properties of Cph1 with PEB adduct expressed in *E. coli* were measured by fluorescence spectrophotometry (Fig. 3D). The wavelength of the excitation maximum was 580 nm and that of the emission maximum was 587 nm. The values obtained with Cph1 phytofluor produced in *E. coli* were comparable to those of the phytofluors constituted in vitro [12]. These results indicated that the expressed bilin reductases from cyanobacterial *pebA* and *pebB* were functional in *E. coli,* and that the *E. coli* cells co-expressing the PEB biosynthesis operon and apo-Cph1 protein produced the holo-Cph1 protein covalently associated with PEB in the cells.

4. Discussion

Expression of recombinant plant apophytochrome has long been reported in model microorganisms such as *E. coli* and yeast [24,25], but it has been a difficult task due to low expression levels and insolubility, especially for full length phytochromes. In a previous study, it was suggested PCB binding to apo-Cph1 increased the total yield of Cph1 in *E. coli* [9]. To see the effect of formation of chromophore adducts on the amount and quality of plant phytochrome expression in *E. coli*, the level of PHYB (N651) fused to the chitin-binding domain were compared in the presence or absence of bilin biosynthetic genes. The total amounts of PHYB (N651) protein with PΦB and PCB adducts were considerably higher than that of apo-PHYB (N651). However, recovery in the soluble fraction did not improve by co-expression of chromophores in the conditions tested here (Fig. 2B, left). The insoluble fractions of PHYB (N651) from the cells co-expressing bilins contain chromophore since the protein were quantitatively detectable by zinc blot assay (Fig. 2B, right). This may be interpreted as inclusion bodies forming after chromophore attachment to PHYB (N651) simply due to over-expression.

We evaluated the assembly of chromophore into phytochrome in *E. coli* by measuring SAR. The values for PCB and PΦB adducts in *E. coli* were ~1.0 after purification by gel filtration chromatography. The values did not change after additional incubation of the purified protein with PCB, and the quantitative binding was also confirmed by zinc blot analysis (Fig. 2C). These results suggested the binding of chromophore in the co-expression system in *E. coli* was efficient. In the system reported here, both phytochrome protein and the enzymes for bilin biosynthesis were simultaneously induced by IPTG. Gambetta and Lagarias reported that PCB biosynthesis before the induction of apoproteins was important for the efficient assembly of apo-Cph1 with the chromophore [9]. The disagreement between these observations might be derived from differences in apophytochromes or constructs for bilin biosynthesis, although detailed analysis of the expression conditions may be needed to reach a clear conclusion.

This production system for phytochrome with PΦB chromophore in *E. coli* will accelerate phytochrome research on photoperception and signaling. Due to the low level of accumulation of phytochromes except for phyA in plants, recombinant phytochromes were used for biochemical assays after assembly with chromophore in vitro. PΦB has been prepared by methanolysis of solvent extracts from rhodophyte and cyanobacteria that contain phycobiliproteins [26], or by enzymatic reduction reaction of BV by PΦB synthase in vitro. PCB has been used as a substitute compound to assemble with apophytochromes in vitro. In this report, we developed a system for the production of large amounts of holophytochrome with PΦB chromophore in *E. coli*. As holoproteins with chromophore are dominant in the culture conditions, the system will

be applicable not only to the biochemical and genetic characterization but also to the tertiary structure analysis of phytochromes.

Protein-based fluorescent probes are a powerful tool to visualize protein localization and behavior in cells. Intense fluorescence from protein–PEB complex including phytofluor [12] and phycobiliprotein known as phycofluor [27] also have potential probes for sensitive detection under fluorescence microscopy. However, exogenous application of chromophore was required for these probes and fluorescence reflected not only protein localization but also the availability of chromophore. A system that produces PEB by transgenic expression of bilin reductases in the cells is potentially advantageous, and applicable to other organisms. Indeed PEB biosynthesis in plants by introducing *pebA* and *pebB* genes has lead to the production of fluorescent phytochrome in plants (Muramoto et al., in preparation).

Recently another engineering application of PCB in *E. coli* was reported to produce light imaging by ''bacteria photograph'' [28]. The metabolic engineering of bilin biosynthesis reported here will also be applicable for bilin-based technology in synthetic biology researches.

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